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Although the functional parameters of microRNAs (miRNAs) have been explored in some depth, the roles of these molecules in viral infections remain elusive. Here we report a general method for global analysis of miRNA function that compares the significance of both overexpressing and inhibiting each mouse miRNA on the growth properties of different viruses. Our comparative analysis of representatives of all three herpesvirus subfamilies identified host miRNAs with broad anti- and proviral properties which extend to a single-stranded RNA virus. Specifically, we demonstrate the broad antiviral capacity of miR-199a-3p and illustrate that this individual host-encoded miRNA regulates multiple pathways required and/or activated by viruses, including PI3K/AKT and ERK/MAPK signaling, oxidative stress signaling, and prostaglandin synthesis. Global miRNA expression analysis further demonstrated that the miR-199a/miR-214 cluster is down-regulated in both murine and human cytomegalovirus infection and manifests similar antiviral properties in mouse and human cells. Overall, we report a general strategy for examining the contributions of individual host miRNAs in viral infection and provide evidence that these molecules confer broad inhibitory potential against multiple viruses.

RNAi | herpesvirus | RNA virus | RNA processing | phosphatidylinositol-3-kinase-Akt signalling

Since the discovery of the first microRNA (miRNA) in *Caenorhabditis elegans*, research in diverse organisms has illuminated the role of this class of small RNA in a wide range of cellular processes (reviewed in ref. 1). MicroRNAs modulate the expression of specific genes by guiding the RNA-induced silencing complex (RISC) to complementary sites within messenger RNAs (mRNAs) (2). This generally serves to down-regulate target genes at specific times, in concert with other regulatory mechanisms in the cell (reviewed in ref. 3). Functional analysis of individual miRNAs suggests diversity in the timing and mechanisms by which they regulate cellular events. For example, some miRNAs promote cellular proliferation, whereas others promote apoptosis (depending on which genes are targeted). Consequently, the expression level of a given miRNA within a cell is expected to be under tight regulatory control, and mechanisms for achieving this control continue to emerge (reviewed in ref. 4).

Viruses require host-cell processes for their survival and have evolved mechanisms for modifying cellular conditions toward an environment conducive to replication while evading recognition and destruction by the host. Herpesviruses are one of the oldest and most successful viral families in this regard. They have coevolved with their hosts for hundreds of millions of years and infect nearly all vertebrate species studied and at least one invertebrate (5). Human cytomegalovirus (HCMV), a member of the β-herpesvirus subfamily that infects a large portion of the world's population (50–90%), is a leading cause of congenital infection (~1% of live births) and a major cause of morbidity in immunocompromised patients. Although mouse and human cytomegaloviruses have diverged over ~80 million years, the pathophysiology of murine CMV (MCMV) in mice is similar to that of HCMV in humans, and the lytic infections result in activation and manipulation of common host-cell signaling cascades (6).

Because miRNAs regulate many aspects of cellular physiology, their expression levels could impact the infection process. It might be expected, therefore, that host miRNA expression is subject to regulation upon infection, by either viral or host factors. Indeed, we and others have previously identified host miRNAs that are down-regulated upon infection by cytomegaloviruses (in some cases within 4 h postinfection) and have demonstrated that these miRNAs exert antiviral properties when overexpressed (7, 8). However, to date, there has been no overlap between results reported with MCMV and HCMV, nor has there been any context with which to interpret the significance of the effect of overexpressing a given miRNA in relation to any other miRNA in the cell. Similarly, various groups have identified mammalian miRNAs that are regulated or implicated in diverse viral infections, but the majority of these studies are generally founded on expression profiling or miRNA target predictions, (reviewed in ref. 9; ref. 10). There is relatively little investigation to date on the functional impact of miRNAs in different infections.

We postulate that specific subsets of host miRNAs are important in controlling the infection process and might be subject to regulation by host and/or viral factors. Although the kinetic parameters of miRNA action are not well-known (e.g., how quickly and reversibly they can modulate a gene or pathway), we expect that viruses with slower replication kinetics (>24 h) might be particularly sensitive to (and exploitive of) changes in host miRNA expression levels. Here we report a combined agonist–antagonist miRNA screening approach that is designed to obtain functional information about mouse miRNAs that impact the lytic phase of herpesviral infections. We further test the breadth of observed antiviral miRNA properties in human cells and against an evolutionarily unrelated RNA virus. Our functional and expression analyses demonstrate that host miRNAs are a tunable and important component of herpesviral infection and provide evidence that these molecules have broad antiviral properties in mouse and human cells against both DNA and RNA viruses.

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Results

Agonist–Antagonist MicroRNA Screen. The combined agonist–antagonist miRNA screening protocol involves analyzing the impact on viral growth of parallel overexpression and inhibition of miRNAs using synthetic mimics or inhibitors (Fig. 1*A*). The miRNA mimics are synthetic RNA duplexes designed to efficiently load a functional mature miRNA strand into RISC. The miRNA inhibitors are single-stranded oligonucleotides that are chemically modified for enhanced binding to mature miRNAs and resistance to nucleolytic cleavage (11). Transfection conditions were optimized to obtain the highest level of transfection efficiency without inducing toxicity (*SI Methods* and Fig. S1). Viral growth is monitored using viruses that encode green fluorescent protein (GFP) reporters. Screening is conducted in a murine fibroblast cell line (NIH 3T3) that supports replication of representatives of all three herpesviral families (α, β, γ): herpes simplex virus 1 (HSV-1), MCMV, and murine gamma herpesvirus 68 (MHV-68) (Fig. 1*B*). Cells are infected at a low multiplicity of infection (MOI; 0.2–0.5), in order that the fluorescent signal detected by ~60–70 h is based on multiple rounds of replication. Therefore, the impact of a miRNA on any stage of the replication cycle is detectable. An assumption in this screening approach is that general miRNA transfection alone does not itself impact viral replication. This is confirmed by control transfections with *C. elegans* miRNA mimics or inhibitors as well as a "RISC-free" small interfering RNA (siRNA), which is chemically modified to be taken up by cells but not incorporated into the RISC:

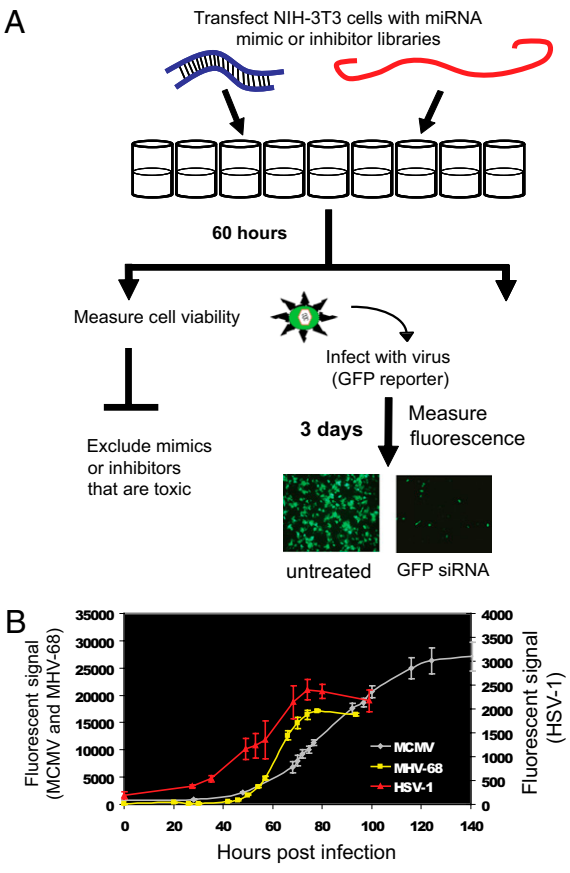

Fig. 1. Combined agonist–antagonist miRNA screening. (*A*) Overview of screening protocol: MicroRNA mimics or inhibitors were reverse-transfected into NIH 3T3 cells (six technical replicates) and incubated for 60 h before cell-viability analysis (n = 3) or infection with GFP virus (n = 3). (*B*) Fluorescent growth curve of GFP-reporter viruses in NIH 3T3 cells; Y1 axis shows values for MCMV and MHV-68 and Y2 shows values for HSV-1; error bars depict SD of three technical replicates.

These reagents do not cause a significant change in the GFP signal, compared with >50% knockdown with siRNAs directed against GFP (Fig. S1). Controls are included in each plate to normalize plate-to-plate variation in fluorescent intensity (*SI Methods* and Fig. S2), and cell-viability assays are performed in parallel to remove from further analysis those reagents that are toxic. In the library analyzed here, a total of 338 pairs of mimics and inhibitors were initially screened (representing 286 unique miRNAs). Twenty-six members of the miRNA mimic library were excluded due to toxicity (*SI Methods*).

Rank Product Statistical Analysis. Mouse miRNAs that negatively impact viral growth are defined in this assay as those that decrease fluorescence in the mimic library and increase fluorescence in the inhibitor library. A common metric for qualifying such "hits" is to accept those mimics or inhibitors that cause a clear difference in fluorescence signal compared with the negative controls (for example, 2 SDs outside the mean of negative controls), reviewed in ref. 12. However, analysis of the mimic and inhibitor data suggests a stark difference in distributions and magnitude of effects of these reagents: The miRNA mimics generally have a much larger effect on the virus, whereas inhibitor results are more subtle (Fig. S2). Although off-targets may be observed with a given mimic in some circumstances, we anticipate that comparison of both mimic and inhibitor increases the chances that observed effects on viral growth are specific (and not idiosyncratic to a particular reagent). The lesser number of inhibitor hits can be partly attributed to the fact that inhibitors only act on expressed endogenous miRNAs and not all miRNAs will be expressed in this cell type.

We therefore opted to identify hits using a robust, nonparametric statistical method that is based on signal ranking and replication between experiments (explained further in *SI Methods*). "Hits" are defined as mimics or inhibitors that result in a high or low fluorescent signal in comparison with the rest of the library, and statistical weight is based on replication between experiments (13). Using this method, "antiviral" miRNAs are defined as those that consistently result in a low fluorescent signal in the mimic library (i.e., overexpression inhibits the quantity of GFP-tagged virus) and a high fluorescent signal in the inhibitor library (due to loss of miRNA-repressor function). Similarly, "proviral" miRNAs are indicated by a high fluorescent signal when overexpressed (as mimics) or low fluorescent signal when inhibited. The impact of these reagents on GFP signal can be visualized as a volcano plot in Fig. 2*A*, which shows a clear correlation between the significance of the rank and the fold change induced by the mimic or inhibitor. Most importantly, the rank-based P values have a more symmetrical distribution, even though the fold-change distribution is quite different, allowing an unbiased selection of significant results from both mimic and inhibitor experiments.

MicroRNAs with Common Anti- or Proviral Properties in Multiple Viruses. Those miRNAs with significant (but opposite) effects on viral growth in mimic and inhibitor datasets are shown in Fig. 2*B*. Excluding miRNAs displaying toxicity, a total of 312 mimics and inhibitors were analyzed. Using the combined datasets for all three viruses, we identified four high-confidence antiviral miRNAs: miR-24, miR-103, miR-199a-3p, and miR-214 (decreased fluorescence in the mimic library, increased fluorescence in the inhibitor library) and three high-confidence proviral miRNAs: miR-30b, miR-30d, and miR-93 (increased fluorescence in the mimic library, decreased fluorescence in the inhibitor library). We then confirmed the screening results in three independent experiments. Five of the miRNAs (miR-24, miR-103, miR-199a-3p, miR-30b, and miR-30d) displayed similar anti- or proviral properties in all three herpesviruses, whereas the other two (miR-93 and miR-214) showed mixed effects (Fig. 3*A*). These miRNAs are perfectly conserved between mouse and human and we therefore extended the analysis to the human CMV virus. As shown in Fig. 3*B*, the same anti- or proviral

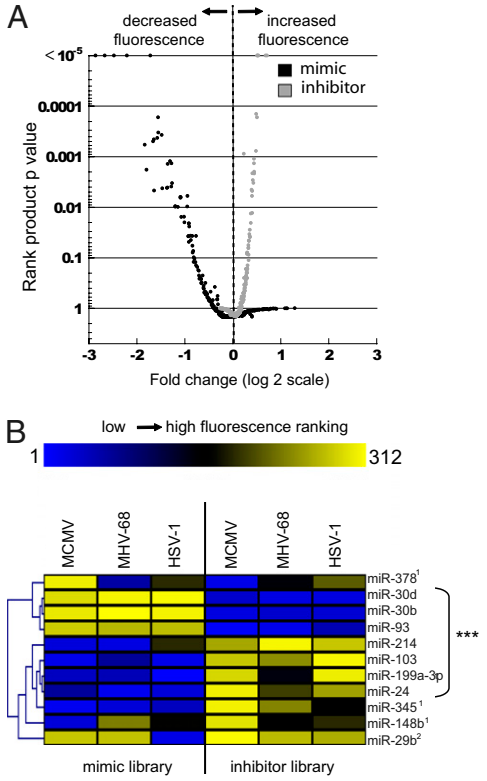

Fig. 2. Rank product results. (*A*) Volcano plot of rank product *P* values based on decreased fluorescent signals in the mimic library and increased fluorescent signals in the inhibitor library. The *x* axis shows log-2 fluorescent changes for mimic or inhibitor (relative to negative controls) and the *y* axis shows the corresponding multiple-testing corrected *P* value. For graphing purposes, we curtailed the minimum *P* value at 0.00001. (*B*) Heat map showing the mean rank in each library (based on 1→312, the number of mimics and inhibitors screened excluding those that were toxic). Only miRNAs that displayed significant (and opposite) effects in mimic and inhibitor libraries are shown. Hits specific to an individual virus are denoted according to 1: MCMV and 2: HSV-1. ***, miRNAs that were identified to be significant when combining datasets for all three viruses.

properties of these miRNAs are observed in human and murine CMV infection (with the exception of miR-93).

Because the MCMV, MHV-68, HSV-1, and HCMV genomes share little sequence similarity, the impact of these miRNAs on viral growth is probably due to regulation of host genes, rather than direct interactions with viral elements. To gain perspective on the breadth of these effects, we also examined Semliki Forest virus (SFV), an arthropod-borne, positive-strand RNA virus of the *Togaviridae* family that is evolutionarily unrelated to the herpesviruses. SFV replicates in the cytoplasm of infected cells such as NIH 3T3, and we have previously used a *Renilla* luciferase (*RLuc*)-expressing SFV to analyze vertebrate immune responses (14, 15). Cells were transfected with mimics or inhibitors and *RLuc* signal was measured 8 h postinfection with SFV (MOI = 0.5). Of the seven miRNAs tested in Fig. 3*A*, four showed anti- or proviral properties based on overexpression, with miR-199a-3p and miR-214 mimics resulting in decreased luciferase signal and miR-30 mimics resulting in increased luciferase signal (as observed with the herpesviruses).

To confirm that the effects observed using the reporter virus are a reliable measure of the quantity of infectious virus, experiments were repeated using the wild-type MCMV virus and quantified with standard plaque assays. Under the same transfection conditions as the screen, the mimics resulted in a *ca.* 10-fold effect on the quantity of infectious virus whereas inhibitors resulted in *ca.* 2- to 5-fold effects (Fig. S3). Growth-curve analysis further demonstrated a 10- to 100-fold decrease in infectious virus based on miR-199a-3p and miR-214 mimics and a 10-fold increase in quantity of virus with the miR-30d mimic at 3 d postinfection (Fig. 3*D*). Plaque assays with MHV-68 and HSV-1 further confirm reporter results, with the miR-199a-3p mimic resulting in >10-fold reductions in infectious virus (in both cases) and the miR-30 mimic resulting in 5- to 10-fold increases (Fig. S3).

It is important to note that this analysis is limited to miRNAs expressed in fibroblasts whose overexpression and inhibition cause the most significant effects on viral growth in three distant herpesviruses. Therefore, a number of real (and perhaps more subtle) functions of miRNAs during the lytic infections are likely to have been missed. In addition, some miRNAs displayed a significant effect on one virus but not on others (e.g., miR-29b is antiviral in HSV-1 and miR-378 is proviral in MCMV; Fig. 2*B*). These may represent miRNAs that interact with specific viral elements or that regulate host genes required by one virus but not the others; this requires further investigation. A full list of rank product results is provided in Dataset S1.

miR-199a/214 Cluster Is Down-Regulated upon Murine and Human Cytomegalovirus Infection. The aforementioned experiments demonstrate that the concentration of specific host miRNAs within a cell can have a significant impact on viral growth capacity. Although the manipulation of such miRNAs could potentially be exploited in a therapeutic context, we expect that those miRNAs most relevant to the infection process might be naturally up- or down-regulated upon infection, either as a host response to viral infection or as a viral strategy to manipulate the host. To determine whether the miRNAs identified as functionally important in the screen are also regulated upon infection, global miRNA microarray analysis was conducted with both the murine and human CMV viruses in murine and human fibroblasts. Although an ideal comparison would involve all three herpesviral subfamilies, we focused on the cytomegaloviruses because the fibroblast cell type used in the screening is a cell type naturally infected by these viruses in vivo. Overall, 47 and 34 probes (out of a total of 648 combined mouse and human probes profiled) displayed a ≥1.5-fold change in response to MCMV or HCMV, respectively (measured 24 and 48 h postinfection, respectively), based on *P* value < 0.05, corrected for false discovery rate (FDR) (*SI Methods* and Fig. S4). Four miRNAs were identified as differentially expressed in both infections: miR-29b*, miR-322, miR-503, and miR-199a-5p (Fig. 4*A* and Dataset S2). This demonstrates overlap in the host miRNAs that are altered in murine and human infection. In particular, all three members of the miR-199a/214 cluster (miR-199a-3p, miR-199a-5p, and miR-214) were significantly down-regulated in either MCMV or HCMV (or both) according to array analysis (Fig. 4*A*). Northern blot analysis confirmed down-regulation of the entire cluster in both mouse and human CMV infection (Fig. 4*B*). This is in contrast to the individual changes previously reported for each virus (e.g., miR-100 is down-regulated upon HCMV infection and miR-27 is down-regulated upon MCMV infection), which we also observed under these conditions (Fig. 4). None of the other miRNAs examined in Fig. 3 were found to be regulated under these conditions by the cytomegaloviruses (Dataset S2). Regulation might, however, occur in other cell types or at time points not analyzed here. The array results reported here overlap with a previous study that identified 23 miRNAs that were down-regulated upon HCMV infection (11 of the 23 previously reported are confirmed here), although a direct comparison cannot be made due to different probe sets and coverage of the microarray platforms (7).

miR-199a-3p Regulation of Host Signaling Networks. Because miR-199a-3p had the largest antiviral effect on MCMV (Fig. 3*D*) and was expressed at a high level in both murine and human cells (Fig. 4), we focus here on genes regulated by this miRNA. Pre-

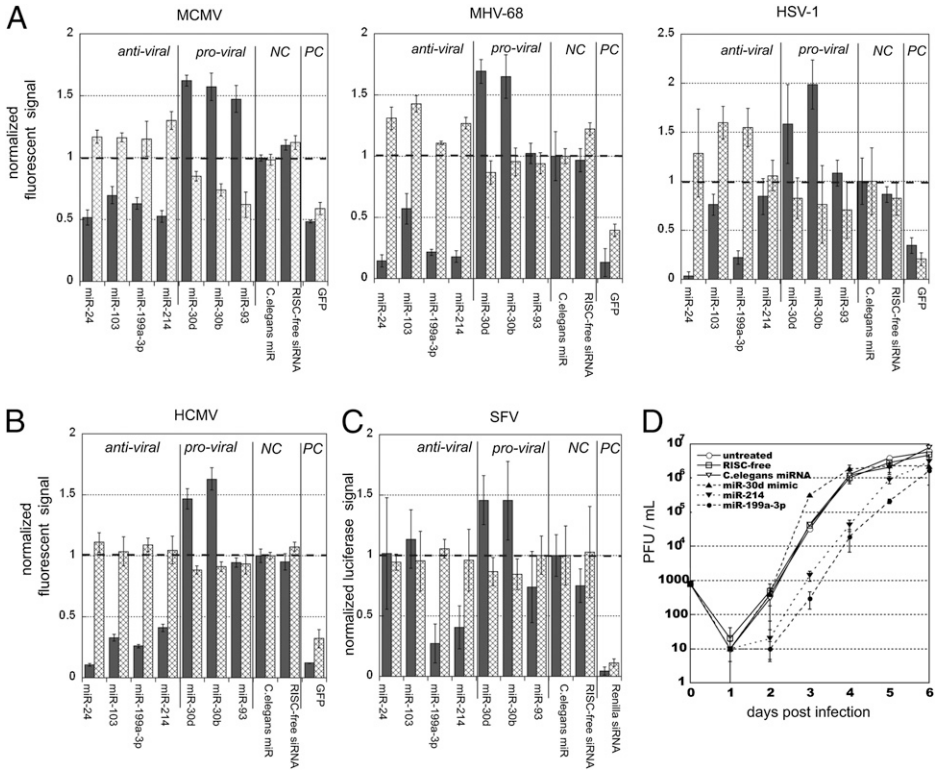

Fig. 3. Validation of anti- and proviral properties of miRNAs in mouse and human cells. (*A*) Normalized fluorescent values of NIH 3T3 cells infected with MCMV [MOI = 0.2, 70 h postinfection (hpi)], MHV-68 (MOI = 0.5, 57 hpi), and HSV-1 (MOI = 0.5, 57 hpi). (*B*) Normalized fluorescent values of MRC-5 cells infected with HCMV (MOI = 0.5, 75 hpi). (*C*) Normalized luciferase values of NIH 3T3 cells infected with SFV (MOI = 0.5, 8 hpi). Cells were transfected with 25 nM miRNA mimics or inhibitors 60 h before infection; values for mimics are shown in gray and inhibitors are in etched gray. NC, negative control; PC, positive transfection control. Data are normalized to values obtained with *C. elegans* mimic and inhibitor. (*D*) MCMV growth curve in NIH 3T3 cells transfected with 25 nM controls compared with miR-199a-3p, miR-214, and miR-30d mimics; cells were infected at an MOI of 0.01; PFU, plaque-forming units. In all experiments, $n = 3$ and error bars indicate SD.

vious studies have demonstrated that miRNAs cause a level of degradation in their targets that can be interrogated by microarray analysis and is often comparable to the extent of change occurring at the protein level (16, 17). Messenger RNA expression levels were examined in cells transfected with the miR-199a-3p mimic versus inhibitor (under the same transfection conditions used in screening; *SI Methods*). To assess whether differences in gene expression between these samples could be explained by direct targeting of miR-199a-3p, an unbiased computational analysis was performed using Sylamer (18). Briefly, Sylamer tests for miRNA-specific effects by searching sorted gene lists for enrichment or depletion of words complementary to miRNA seeds [the first 1–7 or 2–8 nucleotides of a miRNA that largely dictate target interactions (3)]. Genes were sorted based on decreased expression in mimic samples compared with inhibitor samples (left to right, Fig. 5*A*). The only seed-matching words that displayed a significant enrichment in the down-regulated genes corresponded to miR-199a-3p (Fig. 5*A*); this is also the case when examining mimic or inhibitor datasets individually, compared with a negative control, RISC-free siRNA (Fig. S5). Pathway analysis of genes down-regulated in cells transfected with mimic compared with inhibitor (202 in total, based on 1.4-fold cutoff and FDR $P < 0.05$) shows enrichment for pathways normally up-regulated upon infection (19). Specifically, the top 10 significantly enriched pathways include: ERK/MAPK signaling, PI3K/AKT signaling, prostaglandin synthesis, oxidative stress signaling, and viral entry (Table S1). TargetScan (20) identifies 53 of the 202 down-regulated genes as containing seed-matching sites for miR-199a-3p in mouse, and 31 of these also contain seed sites in human (Dataset S3). To account for targets that might be regulated at a level below 1.4-fold, we also list in Dataset S3 genes that are down-regulated in the mimic versus inhibitor samples based on the highest confidence (lowest *P* values), rather than fold changes. From these lists, genes regulated by miR-199a-3p that are known to be relevant to CMV infection are depicted in Fig. 5*B*. These include an integrin, ITGA6, which has been shown to be important for human CMV entry (21); MAP2K1/2 (alias MEK1/2) and PIK3CB, key intermediates in signaling pathways required for viral replication and cell survival (22, 23); and PSTG2 (alias COX2), an enzyme involved in prostaglandin synthesis that is required for effective viral replication and cell-to-cell spread in HCMV (24, 25). All of the regulated genes in Fig. 5*B* are down-regulated by miR-199a-3p mimic and up-regulated by miR-199a-3p inhibitor compared with the negative transfection control, with the exception of ITGA6, which is only up-regulated by the inhibitor (Dataset S3).

Discussion

Small RNAs are involved in many aspects of virus–host interactions from plants to vertebrates, but despite considerable progress in this field a more global view of the miRNA–virus interplay is often missing. Previous reports have shown that disrupting the miRNA machinery can have variable effects on mammalian viruses. For example, some viruses have higher titers in the absence of the miRNA-processing enzyme Dicer, whereas other viruses show the opposite trend (26). We anticipate that interpretation of such experiments (reflecting the net effect of all miRNAs in the cell) is complicated by the fact that host miRNAs have diverse (and opposing) impacts on viruses and, in addition, some viruses encode their own miRNAs (9, 27). Here we describe a general method to study the individual contributions of cellular miRNAs to viral growth and test the hypothesis that specific miRNA subsets are involved in (and consequential to) lytic herpesviral infections. Comparing functional data with miRNA expression analysis, we find that those miRNAs that exhibit the most significant antiviral properties (miR-199a-3p and miR-214; Figs. 2 and 3) are among a small subset of miRNAs down-regulated in both murine and human cytomegalovirus infections (Fig. 4). This demonstrates overlap in the function and regulation of host miRNAs in a murine and human virus and supports the idea that interrogation of miRNA function in animal models is relevant to human disease. A paper published recently reported miR-132 as a miRNA up-regulated in

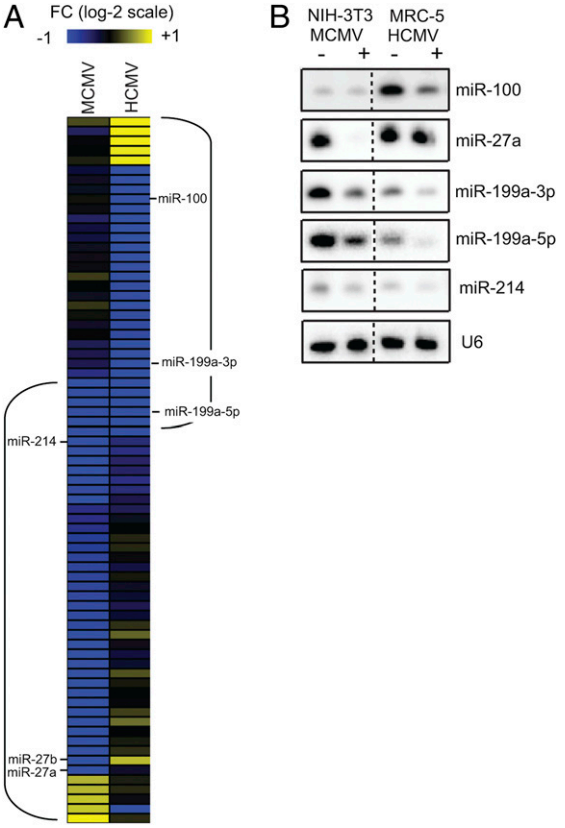

Fig. 4. Changes in miRNA expression upon MCMV or HCMV infection. (*A*) Heat map displaying differential expression of mouse and human miRNAs in MCMV (NIH 3T3 cells, MOI = 3; 24 hpi) or HCMV (MRC-5 cells, MOI = 3; 48 hpi). Color scale is based on log-2 changes in expression; probes identified as significant in each dataset are bracketed (left, MCMV; right, HCMV), with overlap depicted. FC, fold change. (*B*). Northern blot analysis of total RNA isolated from mouse fibroblast NIH 3T3 cells infected with MCMV at MOI = 3 (24 hpi) or human fibroblast MRC-5 cells infected with HCMV at MOI = 3 (48 hpi). Dashed lines indicate a gap between lanes that was removed in making the image.

Kaposi's sarcoma-associated herpesvirus infection that facilitates viral replication and regulates antiviral innate immunity (28). This further supports the concept that some mammalian hosts and viruses might have evolved mechanisms to regulate individual miRNAs (or specific subsets) upon infection, rather than targeting the global miRNA machinery. Further work in other infection systems and cell types is required for a more global perspective of the antiviral miRNAs identified here, as well as for miR-132, which was excluded from our analysis due to nonspecific effects in the mimic library (*SI Methods*).

We previously reported rapid down-regulation of miR-27a and miR-27b in MCMV infection and speculated that this regulation might reflect antiviral functions of these miRNAs (8). Neither miR-27a nor miR-27b is among the most significant antiviral miRNAs identified here. However, our analysis is limited to those miRNAs that display the most dramatic (and opposite) effects on viral growth when overexpressed and inhibited in fibroblasts. Although this allows for high confidence in the hits identified, it may miss more subtle, but real, effects of other miRNAs and will be cell-type-specific. An underlying assumption is also that overexpression of a miRNA increases the extent to which it represses its targets; this depends on the concentrations of miRNAs and targets, which will be variable in different cells. Further understanding of miRNA biology is required to shed light on the generality of this assumption. Although miR-27a and miR-27b are not scored as significant in the mimic libraries (according to rank product analysis), they are among the most significant antiviral miRNAs when examining the combined inhibitor datasets for all three viruses, along with miR-24 and miR-23b (Dataset S1), with which they are coregulated.

We focus here on miRNAs with common properties in multiple herpesviral infections; however, there are notable differences in the screening results with different viruses (for example, miR-378 is proviral in MCMV but not the other infections; Fig. 2*B*). Viruses can evolve miRNA binding sites in their genomes (29), and it is possible that differences observed here are related to sequence-specific miRNA interactions with viral elements. Given the expected frequency of miRNA target sites in a ~200,000-base-pair herpesviral genome (on average, a 7-mer seed site will occur every ~16,384 nt), investigations in this area will require extensive biochemical validation.

To date, the therapeutic capacity of miRNA manipulation in viral infection has focused primarily on blocking interactions between a host miRNA and a viral sequence (for example, miRNA-122/HCV) (29, 30). Here we find that miR-199a-3p leads to decreased viral growth in all three herpesviral subfamilies as well as an unrelated positive-strand RNA virus. Although we cannot eliminate the possibility that the antiviral properties are at least partly

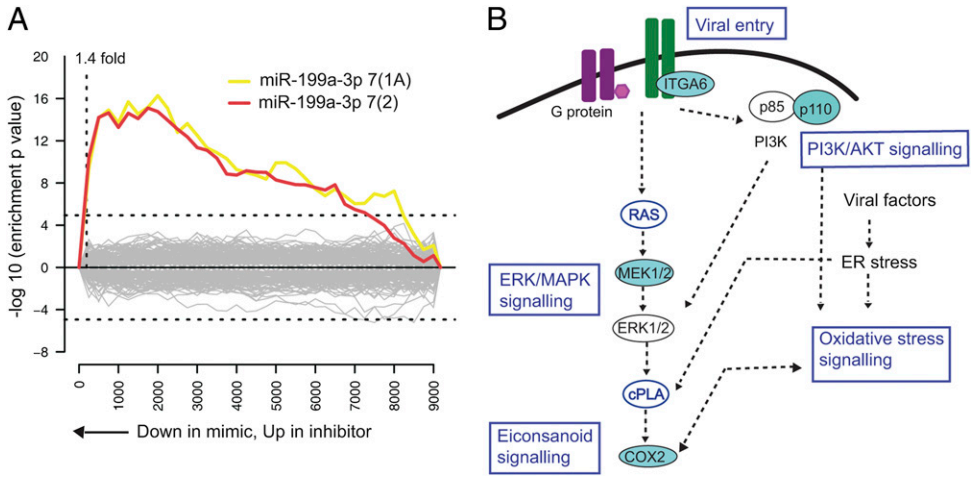

Fig. 5. Cellular networks modulated by miR-199a-3p. (*A*) Plot of the significance of enrichment of all 7-mer words complementary to known mouse miRNA seed regions in the mRNA array dataset, comparing mimic against inhibitor results. The *x* axis represents all of the genes with an annotated 3′UTR sequence, sorted starting from the most down-regulated by the miR-199a-3p mimic (equivalent to the most up-regulated by the inhibitor). The *y* axis represents the $-\log_{10}$ transformed *P* values, with positive values denoting enrichment and negative values denoting depletion. Each gray line represents a single 7-mer, tested for enrichment and depletion at every 250 genes. Two seed-matching 7-mers for miR-199a-3p, 7-mer(1A) = CTACTGA and 7-mer(2) = ACTACTG, are highlighted in gold and red, respectively. Horizontal dotted lines represent Bonferroni-corrected *P* value significance thresholds of 0.01. (*B*) Pathways regulated by miR-199a-3p that are known to play a role in CMV infection. Selected genes down-regulated by miR-199a-3p are shown in blue circles (and are shaded if they also represent a predicted target); human alias gene names are used.

based on interactions with viral elements [as suggested in HCV (31)], we identify several host pathways regulated by this miRNA that are activated and required by multiple viruses. Specifically, ERK/MAPK signaling, prostaglandin synthesis, oxidative stress signaling, and PI3K/AKT signaling are all down-regulated by miR-199a-3p (Fig. 5 and Table S1). The regulation of these pathways by miR-199a-3p is consistent with previous (although diverse) reports on miR-199a-3p, a stress-inducible miRNA up-regulated during cardiac hypertrophy (32) that is also reported to target MET (33), a transmembrane tyrosine kinase receptor associated with invasive growth of tumors, and COX2 (34), an enzyme involved in prostaglandin synthesis. Our findings suggest links between this miRNA and viral infection. Greater understanding of the role of miR-199a-3p in normal and disease biology will shed light on the best strategies for exploiting its antiviral properties. It is notable that the other miRNA with which it is coregulated, miR-214, is among the four miRNAs identified here as broadly antiviral, (Figs. 2 and 3) and may function in concert with miR-199a-3p.

Given the large number of potential targets of any given miRNA [on average, in the hundreds (35)], and in particular those described in this study, it may not be that one specific target (or even a handful of targets) sufficiently explains a miRNA-related phenotype. We anticipate that understanding miRNA function may require a "whole is greater than the sum of the parts" approach (involving multiple targets), which might also hold true in developing more effective antiviral therapeutics.

Methods

For a full description of the methods used here, see *SI Methods*. The miRNA mimic and inhibitor libraries are based on miRBase 8.2. The engineered reporter viruses have been described elsewhere (14, 36–39). MicroRNA microarrays are based on Exiqon miCURY probe set version 8.1. Pathway analysis was carried out using Ingenuity software and targets were predicted based on targetscan.org (TargetScanHuman version 5.1) (20).

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1. Chang TC, Mendell JT (2007) microRNAs in vertebrate physiology and human disease. *Annu Rev Genomics Hum Genet* 8:215–239.
2. Lee RC, Feinbaum RL, Ambros V (1993) The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 75:843–854.
3. Bartel DP (2009) MicroRNAs: Target recognition and regulatory functions. *Cell* 136: 215–233.
4. Winter J, Jung S, Keller S, Gregory RI, Diederichs S (2009) Many roads to maturity: MicroRNA biogenesis pathways and their regulation. *Nat Cell Biol* 11:228–234.
5. Davison AJ (2002) Evolution of the herpesviruses. *Vet Microbiol* 86:69–88.
6. Krmpotic A, Bubic I, Polic B, Lucin P, Jonjic S (2003) Pathogenesis of murine cytomegalovirus infection. *Microbes Infect* 5:1263–1277.
7. Wang FZ, et al. (2008) Human cytomegalovirus infection alters the expression of cellular microRNA species that affect its replication. *J Virol* 82:9065–9074.
8. Buck AH, et al. (2010) Post-transcriptional regulation of miR-27 in murine cytomegalovirus infection. *RNA* 16:307–315.
9. Umbach JL, Cullen BR (2009) The role of RNAi and microRNAs in animal virus replication and antiviral immunity. *Genes Dev* 23:1151–1164.
10. Parameswaran P, et al. (2010) Six RNA viruses and forty-one hosts: Viral small RNAs and modulation of small RNA repertoires in vertebrate and invertebrate systems. *PLoS Pathog* 6:e1000764.
11. Vermeulen A, et al. (2007) Double-stranded regions are essential design components of potent inhibitors of RISC function. *RNA* 13:723–730.
12. Birmingham A, et al. (2009) Statistical methods for analysis of high-throughput RNA interference screens. *Nat Methods* 6:569–575.
13. Breitling R, Armengaud P, Amtmann A, Herzyk P (2004) Rank products: A simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. *FEBS Lett* 573:83–92.
14. Kiiver K, et al. (2008) Properties of non-structural protein 1 of Semliki Forest virus and its interference with virus replication. *J Gen Virol* 89:1457–1466.
15. Mankouri J, et al. (2010) Optineurin negatively regulates the induction of IFNβ in response to RNA virus infection. *PLoS Pathog* 6:e1000778.
16. Baek D, et al. (2008) The impact of microRNAs on protein output. *Nature* 455:64–71.
17. Selbach M, et al. (2008) Widespread changes in protein synthesis induced by microRNAs. *Nature* 455:58–63.
18. van Dongen S, Abreu-Goodger C, Enright AJ (2008) Detecting microRNA binding and siRNA off-target effects from expression data. *Nat Methods* 5:1023–1025.
19. Fortunato EA, McElroy AK, Sanchez I, Spector DH (2000) Exploitation of cellular signaling and regulatory pathways by human cytomegalovirus. *Trends Microbiol* 8: 111–119.
20. Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120: 15–20.
21. Feire AL, Koss H, Compton T (2004) Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain. *Proc Natl Acad Sci USA* 101:15470–15475.
22. Johnson RA, Wang X, Ma XL, Huong SM, Huang ES (2001) Human cytomegalovirus up-regulates the phosphatidylinositol 3-kinase (PI3-K) pathway: Inhibition of PI3-K activity inhibits viral replication and virus-induced signaling. *J Virol* 75:6022–6032.
23. Johnson RA, Ma XL, Yurochko AD, Huang ES (2001) The role of MKK1/2 kinase activity in human cytomegalovirus infection. *J Gen Virol* 82:493–497.
24. Schröer J, Shenk T (2008) Inhibition of cyclooxygenase activity blocks cell-to-cell spread of human cytomegalovirus. *Proc Natl Acad Sci USA* 105:19468–19473.
25. Zhu H, Cong JP, Yu D, Bresnahan WA, Shenk TE (2002) Inhibition of cyclooxygenase 2 blocks human cytomegalovirus replication. *Proc Natl Acad Sci USA* 99:3932–3937.
26. Otsuka M, et al. (2007) Hypersusceptibility to vesicular stomatitis virus infection in Dicer1-deficient mice is due to impaired miR24 and miR93 expression. *Immunity* 27: 123–134.
27. Pfeffer S, et al. (2004) Identification of virus-encoded microRNAs. *Science* 304: 734–736.
28. Lagos D, et al. (2010) miR-132 regulates antiviral innate immunity through suppression of the p300 transcriptional co-activator. *Nat Cell Biol* 12:513–519.
29. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P (2005) Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA. *Science* 309:1577–1581.
30. Lanford RE, et al. (2010) Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* 327:198–201.
31. Murakami Y, Aly HH, Tajima A, Inoue I, Shimotohno K (2009) Regulation of the hepatitis C virus genome replication by miR-199a. *J Hepatol* 50:453–460.
32. van Rooij E, et al. (2006) A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. *Proc Natl Acad Sci USA* 103: 18255–18260.
33. Kim S, et al. (2008) MicroRNA miR-199a* regulates the MET proto-oncogene and the downstream extracellular signal-regulated kinase 2 (ERK2). *J Biol Chem* 283: 18158–18166.
34. Chakrabarty A, et al. (2007) MicroRNA regulation of cyclooxygenase-2 during embryo implantation. *Proc Natl Acad Sci USA* 104:15144–15149.
35. Friedman RC, Farh KK, Burge CB, Bartel DP (2009) Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 19:92–105.
36. Dutia BM, et al. (2004) Identification of a region of the virus genome involved in murine γherpesvirus 68-induced splenic pathology. *J Gen Virol* 85:1393–1400.
37. Sun A, et al. (2004) Immediate-early expression of the herpes simplex virus type 1 ICP27 transcript is not critical for efficient replication in vitro or in vivo. *J Virol* 78: 10470–10478.
38. Angulo A, et al. (2000) Identification of a boundary domain adjacent to the potent human cytomegalovirus enhancer that represses transcription of the divergent UL127 promoter. *J Virol* 74:2826–2839.
39. Angulo A, Ghazal P, Messerle M (2000) The major immediate-early gene ie3 of mouse cytomegalovirus is essential for viral growth. *J Virol* 74:11129–11136.